ANTIMICROBIAL ACTIVITY OF PROCESSED HONEY OF SOME RENOWNED COMPANY FOUND IN CHATTOGRAM CITY AGAINST *Staphylococcus aureus* AND *Escherichia coli*

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Roll No.: 0119/04

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A thesis submitted in the partial fulfilment of the requirements for the degree of

Master of Science in Food Chemistry and Quality Assurance

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This is to certify, that we have examined the above Master's thesis and have found that is complete and satisfactory in all respects, and that all revisions required by the thesis examination committee have made

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DEDICATED TO MY RESPECTED AND BELOVED FAMILY AND TEACHERS

PLAGIARISM VERIFICATION

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Abbreviations

%	Percentage
°C Degree	Celsius
AFB	American Foulbrood
a_w	Water activity
BC	Before Christ
CFU	Colony Forming Unit
CLSI	Clinical & Laboratory Standards Institute
DNA	Deoxyribonucleic Acid
etc	Et cetera
g	Gram
GC-MS	Gas Chromatography-Mass Spectrometry
H_2O_2	Hydrogen peroxide
HMF	Hydroxymethylfurfural
HPLC	High Performance Liquid Chromatography
KDa	Kilodaltons
kg	Kilogram
MBC	Minimum Bacterial Concentration
MGO	Methylglyoxal
MIC	Minimum Inhibitory Concentration
min	Minute
ml	Milliliter
mm	Millimeter
MRJPs	Major royal jelly proteins
NO	Nitric oxide
ORAC	Oxygen Radical Absorbance Capacity
P	Vapor pressure
p^0	Vapor pressure of water
pH	Potential of Hydrogen
PRTC	Poultry Research and Training Centre
ROS	Reactive Oxygen Species
SPE	Solid phase extraction
TLC	Thin Layer Chromatography
v/v	volume by volume
VOCs	Volatile organic compounds
w/v	Weight by volume
w/w	Weight by weight
ZOI	Zone of inhibition

Abstract

The research work was conducted to evaluate the antimicrobial effect of 10 honey samples which are of imported renowned company in Chattagram on *Escherichia coli* and *Staphylococcus aureus.* The antimicrobial effects were assayed by disc diffusion method in Mueller Hinton agar plate. The effects were evaluated by measuring the zone of inhibition around the discs. Antimicrobial activity of the honey samples was carried out individually where 5 discs of individual samples placed on an agar plate. No noticeable variations in the antimicrobial activity of 10 types of honey were observed. There was no zone of inhibition of honey against *Escherichia coli* and *Staphylococcus aureus*. This research work revealed that processed honey doesn't show any zone of inhibition against *Escherichia coli* and *Staphylococcus aureus.*

Keywords: Antimicrobial activity, honey, *Escherichia coli*, *Staphylococcus aureus*, disc diffusion method.

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Chapter 1: Introduction

Antimicrobial activity is the process of eliminating or suppressing germs that cause illness. A vast range of bacteria are resistant to the antimicrobial effects of various plants, fruits, and spices. Antimicrobial substances can be antiviral, antifungal, or antibacterial. Numerous foods have been demonstrated in studies to exhibit broad-spectrum antibacterial effects against a range of harmful bacteria, including anaerobes, aerobes, and gram-positive and gram-negative bacteria (Mundo et al., 2004). *Staphylococcus aureus* and *Escherichia coli* are the most widespread human pathogenic bacteria that cause wound infections (Doglas et al., 2004).

One of the oldest traditional treatments still used today to treat microbial diseases is honey. Additionally, it is recognized as a successful native antibiotic using an antibacterial to treat burns and wounds (Brudzynski, 2006). Researchers get concerned about various forms of honey with antibacterial property as a result (Mullai and Menon, 2007). From 2100 to 2000 BC, honey was utilized to treat illnesses. It is common knowledge in current science that Aristotle (384–322 BC) said that pale honey was "excellent for painful eyes and bruises" (Mandal and Mandal, 2011; Vallianou et al., 2014). Honey's antibacterial effects have been thoroughly studied and documented. It has also been utilized as a wound healing accelerator from ancient times. Its effectiveness in promoting wound healing has been demonstrated and frequently documented (Vallianou et al., 2014). Honey's antibacterial properties were initially identified by Dustmann in 1892 (Dustmann, 1989). Even in modern medicine, honey is still used as a remedy. In a few institutions, it is used mostly in the clinical management of burns, bedsores, and ulcers, wounds from surgery, and traumas. Honey's antibacterial characteristics may be helpful in combating germs like those that have become resistant to a variety of antibiotics. Hospital wound sepsis is primarily caused by *Staphylococcus aureus* (Armstrong and Otis, 1995). Additionally, honey is an excellent topical wound dressing agent for wound infections, burns, and surgical infections (Betts and Molan, 2002). Natural, unheated honey when tested against pathogenic bacteria, oral bacteria, and germs that cause food to spoil, has been shown to have some broad-spectrum antibacterial effect was observed. (Basson et al., 1994 and Mundo et al., 2004). According to recent research, honey has an inhibitory impact on about 60 kinds of bacteria, including

gram-positive and gram-negative, aerobes and anaerobes. It also kills or inhibits the growth of some dangerous vegetative microorganisms (Chick and Shin, 2001). According to some studies, honey has antibacterial properties that can fight off bacteria like *Escherichia coli, Campylobacter jejuni, Salmonella entercolitis, Shigella dysenteriae* (Adebolu, 2005), *Mycobacterium* (Asadi-Pooya et al.,2003), Methicillin-resistant *Staphylococcus aureus* and Vancomymin-resistant *Enterococci* (Cooper et al.,1999 & 2002 and Al-waili et al.,2005), Common Gastrointestinal Pathogenic Bacteria (Lin et al.,2011), Streptococcus *pyogenes* biofilms (Maddocks et al., 2012). Additionally, it has been shown that honey exhibits antifungal properties against the majority of prevalent dermatophytes (Brady et al., 1997) as well as certain yeasts, *Aspergillus*, and *Penicillium* species (Quinn et al., 1994). There have also been reports of the honey's anti-*Candida* properties (Ahmed et al., 2012). Honey may have therapeutic powers due to a variety of its physical and chemical characteristics (Snow and Manley-Harris, 2004). In terms of total carbohydrates, honey contains about 82.4 percent (38.5 percent fructose, 31.0 percent glucose, and 12.9 percent from maltose, sucrose, and other sugars (Khan et al., 2007; Vallianou et al., 2014). Depending on the floral source, the grazing areas, the climate where the bees were raised, and the nectar's natural composition, the biological characteristics of honey can vary (Abd-El Aal et al., 2007). Blood can include more iron, antioxidants, and rare elements when honey is consumed (Theunissen et al., 2001).

In comparison to regularly used antibacterial treatments, honey exhibited a more pronounced inhibitory impact (85.7 percent) on Gram negative bacteria (*Pseudomonas aeruginosa*, *Enterobacter* spp., and *Klebsiella*), according to a study by Abd-El Aal et al. in 2007. When compared to the usage of antibiotics alone, Gram-positive, methicillin-resistant Staphylococcus aureus showed a 100% inhibition. When honey and antibacterial drugs were combined, they worked in concert against both Gram-negative and -positive bacteria. Honey naturally inhibits the growth of most bacteria, as well as many yeasts and molds, due to its little water activity and more osmotic pressure. Honey also has innate antibacterial characteristics. Osmosis should suck water from the wound into the honey if honey is given topically to wounds, assisting in the drying of the damaged tissue and reducing bacterial growth One hypothesis for its activity is that it can cause hydrogen peroxide to be produced by the enzyme glucose oxidase, which is originated from bees.

Although honeys would likely keep a water activity low enough to hinder the growth of the majority of bacteria when diluted with water absorbed from wounds. Honey has acidic properties; its pH ranges from 3.2 to 4.5. The bees' division of glucose oxidase, which catalyzes the glucose to gluconic acid conversion, results in the gluconic acid formation in honey. Numerous pathogenic bacteria can be inhibited by the low pH alone, and at the very least this might be sufficient for topical treatments to exert an inhibitory effect (Molan, 1995). When used against pathogens, hydrogen peroxide possesses antibacterial effects (Snowdon and Cliver, 1996). This well-known antiseptic, when used in small amounts, inhibits the growth of infectious germs, promotes the healing of wounds (Molan, 2001), and increases lymphocytic and phagocytic activity in peripheral blood. (Tonks et al., 2001). Other characteristics of honey, including its Poor protein content, high concentration of reducing sugars, high carbon to nitrogen ratio, viscosity/anaerobic atmosphere, low redox potential, and other chemical agents/phytochemicals are also responsible for contributing to its antibacterial activity (Honey, 2002). Additionally, honey has been used to reduce the length of diarrhea in people with bactericidal gastro-enteritis brought on by bacterial infection (Haffejee and Moosa, 1985). However, honey contains other crucial beneficial properties that are less affected by storage circumstances (Cooper et al., 2002).

Before the invention of antibiotics, the only treatment option was using both conventional medication and natural remedies to treat illnesses such as cough, fever, catarrhal disorders, gastrointestinal ailments, etc. This practice dates back to the dawn of humankind. Various plant extracts and treatments requiring antibacterial properties have been employed (Jawad, 2011). Antibiotics provided a cure for bacterial diseases; sadly, due to their overuse and abuse, their effectiveness has declined over time.

The development of novel antibiotic compounds is difficult, and it necessitates significant financial investments corresponding to the length of the testing period as well as careful consideration of any possible negative consequences that can arise from their use. In recent years, medical professionals have seen an increase in infections and the creation of strains that are resistant to a number of antibacterial substances, primarily as a result of the misuse of these drugs (Aggad and Guemour, 2014). The usage of natural compounds has been taken into consideration as one of the potential solutions. Alternative antimicrobial

techniques, such as using plants and plant-based items like lemon, honey, garlic, ginger, etc. to address this issue, are currently receiving increased attention.

Aims and Objectives

The present study aimed to evaluate the antibacterial activity of 10 honey samples collected from a retail shop in Chattogram District which are imported from different countries against *Escherichia coli* and *Staphylococcus aureus.*

Chapter 2: Review of literature

2.1 Honey

Honey is a sweet, viscid substance that different types of bees collect from the nectar of flowers in their honey sacs. It contains 82.0 percent carbohydrates (sucrose, fructose, and maltose), 0.3 percent protein, 17.0 percent water, and 0.7 percent minerals, vitamins, and antioxidants (National Honey Board). Honey also has a number of minerals and vitamins, particularly the B complex and vitamin C, in addition to carbohydrates. While honey contains a variety of minerals, such as calcium, copper, iron, magnesium, manganese, phosphorus, potassium, and zinc. Ascorbic acid, pantothenic acid, niacin, and riboflavin are a few vitamins that can be found in honey. (Ajibola et al.,2012). Honey contains at least 181 different components (Bogdanov et al.,2008). Aside from these components, honey also contains proteins, antibiotic-rich inhibine, amino acids, and phenol antioxidants. Other bioactive components include proteins, organic acids, carotenoid-derived chemicals, nitric oxide (NO) metabolites, flavonoids, phenolic components, and organic acids.

According to some research, some honey types include kynurenic acid, a tryptophan metabolite with neuroactive activity, which can be a factor in the antibacterial characteristics of the honey (Beretta, 2010). Honey has also been found to include enzymes such glucose oxidase, diastase, invertase, phosphatase, catalase, and peroxidase. Honey contains significant amounts of ascorbic acid, catalase, peroxidase, flavonoids, phenolic acids, and carotenoids, which all function as antioxidants (Bosi and Battalglini, 1978).

For the benefit of human health, consuming honey can improve the total plasma antioxidant and reducing capacity is increased. Honey is widely produced and consumed in Bangladesh. With 334 plant species, Sundarbons, the biggest mangrove forest in the world, is a perfect habitat for gigantic honey bees (Apis dorsata) and honey collectors.

2.2 Chemical composition

2.2.1 Water content and water activity (a_w)

Honey's water activity depends on a number of variables, including the nectar's botanical and geographic origin, soil and climate conditions, harvesting season, nectar flux intensity, maturation level, beekeeper manipulation during harvest, and extraction, processing, and storage conditions. In honeys from various plant sources, moisture concentrations might differ. Since some plants have a high natural water content such as heather, clover and strawberry tree, they produce honey with a high-water activity (Persano-Oddo, Piazza, Sabatini, & Accorti, 1995).

A quality factor that affects the shelf life of honey is moisture. According to Ortiz-Valbuena, Fernandez-Maeso, and De La Torre (1996), when the storage cells are completely covered in beeswax, the water percentage in honey is appropriate. Honey's moisture typically ranges from 13 to 25 percent, with 17 percent being the ideal level (Simal, Huidobro, & Araquistain, 1983).

It is challenging to handle and prepare honeys with extremely low moisture levels. However, honeys with moisture levels above 18% are more susceptible to ferment because the osmotic pressure of sugar is insufficient to prevent the growth of osmophilic (sugar-tolerant) yeast (Bogdanov & Martin, 2002). If there are fewer yeasts that cause honey to ferment, the moisture content of the honey will be higher (Piana et al., 1989). Other characteristics of honey, including color, crystallization, viscosity, flavor, and density, are also influenced by its water concentration. Because honey has a high hygroscopicity, it is essential to avoid absorbing moisture from the environment during manufacturing and packing. (White, 1975). The amount of water that is accessible to microorganisms in food is measured by water activity (a_w). Instead of the water content being the determining factor for bacterial deterioration, sugar locks up some of the water and prevents it from being available for microbial growth. The relationship between the vapor pressure of food water (p) and the pure water vapor pressure (p_o) at the same temperature is known as water activity. Pure water has a water activity of 1, and each addition of a chemical that fixes water results in p less than p_o. Because of this, the water activity is never more than 1 (Gleiter, Horn, & Isengard, 2006).

Water activity of honey typically ranges from 0.49 to 0.65, though it is possible to exceed 0.75 for some honeys (Costa et al., 2012). The average water activity needed for the growth of microorganisms is 0.90 for bacterial growth, 0.80 for yeast, and 0.70 for mold growth. Aw readings below 0.60 will prevent the osmophilic yeasts that induce fermentation of honey from growing (Bogdanov, 2011 b;). Once more, parameters including temperature, pH, carbon dioxide and oxygen content, as well as the presence of inhibitory chemicals, have an impact on how bacteria develop. Glucose content and the glucose/fructose ratio, honey crystallization, and environmental factors all affect water activity (Gleiter et al., 2006). Given that honey either accumulates or loses moisture when exposed to varying ambient relative humidity values, honey's water activity often predicts moisture exchange with the environment (Chirife, Zamora, & Motto, 2006;).

Additionally, the water activity of a particular honey's crystallized state is greater than that of its liquid condition. This occurs as a result of the water related to glucose being released during the crystallization process. Blossom honeys had lower water activity in the liquid form than honeydew honeys with the same water content (Gleiter et al., 2006).

However, they were unable to detect any appreciable differences in the water activities of various honey species after they were crystallized. Some researchers have discovered strong links between the moisture content and water activity of honey (Cavia et al., 2004). Water activity, rather than moisture, is a better marker of the quality of honey because it shows the amount of free water present, which is ultimately utilised by microbes to promote fermentation (Bogdanov, 2011b).

2.2.2 Sugar content

As a naturally occurring supersaturated solution of sugar, honey primarily consists of carbohydrates, which make up around 95% of the dry matter (Bogdanov et al., 2008). The amount of sugar in honey affects its most significant physical, chemical, and nutritional characteristics, including sweetness, viscosity, granulation, hygroscopicity, energy value and specific rotation.

Furthermore, a key honey antibacterial component is the osmotic pressure caused by high sugar concentration (Jeddar et al., 1985). Honey has been consumed as a culinary item for ages as a sweetener and a source of energy for people. Monosaccharides (hexoses) glucose (23–38%) and fructose (32–44%) make up the majority of the sugars in honey. Other

monosaccharides, like galactose, have been found in very minute levels (Val et al., 1998). Honey bees synthesize these sugars throughout the ripening phase by converting nectar sucrose in the presence of the enzyme invertase from the salivary glands of the bees. Additionally, invertase contains transglucosilation activity, which converts monosaccharides into complex sugars (White & Maher, 1953). Trisaccharides and tetrasaccharides are probable α-glucosyl derivatives of the principal disaccharides and trisaccharides, respectively, making the primary disaccharides in honey α-glucosyl derivatives of monosaccharides (Ruiz et al., 2010). In the digestive system of the plant-eating insects (Hemiptera, primarily aphids) that produce honeydew, some additional disaccharides and trisaccharides possessed in honey may be created by microbial action and enzymatic reactions (Kolayli et al., 2012). Maltose, sucrose, trehalose, lactose,gentiobiose, turanose,isomaltose, raffinose, erlose, maltotriose,kojibiose, melezitose, panose, isomaltotriose, and maltotetraose are just a few examples of the more than 45 disaccharides, trisaccharides, and other oligosaccharides and polysaccharides (Ruiz et al., 2010; Val et al., 1998). The most significant disaccharides in honey are maltose (7%) and sucrose (1%) (Shin & Ustunol, 2005). Because not all of the sucrose in nectar or honeydew is digested by the invertase enzyme, there are high levels of sucrose present in honey. High sucrose content in honeys is associated to its botanical origin, honey immaturity, high nectar flux, or artificial bee feeding. The classification of unifloral honeys can be based on the varying amounts and types of carbohydrates found in samples from various vegetal sources. Carbohydrate concentrations were used to distinguish blossom and honeydew honeys, because honeydew honeys contain lower levels of monosaccharides, higher levels of trissacharides (mainly melezitose, erlose, raffinose and maltotriose), as well as higher levels of other oligosaccharides than blossom honeys (Kolayli et al., 2012 ;). It was suggested that ratios between some of these components, as well as sugar amounts, might make good markers for determining the genuineness of honey (Nozal et al., 2005). The composition of honey sugars can vary depending on the freshness of the honey and how it is stored.

Due to acid reversion and enzymatic activity (White, 1979), the number of monosaccharides reduces during storage while the amount of oligosaccharides increases. Because the sugar concentration is related to how ripe the honey is and can indicate

possible adulteration (Belay et al., 2013), honey quality standards regulations define minimum limitations for the sum of fructose and glucose as well as maximum limits for sucrose (Governments of Argentina, 2008; Brazil, 2000). It is important to consider that these parameters also can vary according to botanical origin and processing.

2.2.3 Proteins and Amino acids

Honey contains protein that is derived from both plants and bees' salivary glands (honeydew nectar, and mainly pollen). Nearly 20 various nonenzymatic proteins, including albumins, globulins, proteases, and nucleoproteins, have been found in honey, many of which are present in all honeys (Doner, 2003). Typically, protein present in honey ranges from 0.1 to 0.5 percent (Won et al., 2009).

Honey contains 18 different amino acids, with proline accounting for between 50 and 85% of the entire profile. Some amino acids, such as cystine, arginine, and tryptophan, are distinctive to certain varieties of honey (Anklam, 1998). Minor portion of these proteins are enzymes.

2.2.4 Vitamins and Minerals

Honey contains very little ascorbic acid and very little of the B vitamins (niacin, riboflavin, pantothenic acid, folic acid, and vitamin B6). Unprocessed honey have a variety of minerals, including iron, potassium, chromium, zinc,manganese, calcium,magnesium, and phosphorus.

2.2.5 Volatile compounds

Volatiles are those organic chemicals which have more vapour pressure at standard room temperature. In honey, almost 600 volatile organic compounds (VOCs) have been found. Aldehydes, ketones, acids, alcohols, esters, hydrocarbons, and cyclic compounds are just a few of the seven primary classes of volatile components that have been previously identified in honey (Kaskonien and Venskutonis, 2010; Loh et al., 2011). VOCs, such as ()-3- Hydroxy-4-phenyl-2-butanone and (+)-8-hydroxylinalool, have been found in honey. These VOCs exhibit strong antibacterial action against bacteria such *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and pathogenic fungus *Candida albicans* (Melliou and Chinou, 2011).

The VOCs contain the potential to be used as natural therapies to treat a variety of pathogenic microbial organisms even though they are present in minimal concentrations and may contribute to overall antibacterial activity. Low concentrations of VOCs in honey affect its sensory qualities, including flavor, scent, color, and texture, which are all influenced by the plants and flowers types that bees visit (Loh et al., 2011).

2.2.6 Hydroxymethylfurfuraldehyde (HMF)

Honey contains hydroxymethylfurfuraldehyde (HMF) in trace amounts as well. One aspect of honey's quality and marketing is HMF. It has been determined that even fresh honeys do contain trace amounts of HMF (Zappala et al., 2005), which could easily be elevated if the honey is stored in moderate or high temperatures. As a result, in order to keep HMF levels to a minimum, honey must be kept in a refrigerator or another cool environment. (White, 1975). HMF is considered to be the adulteration indicator of honey and could be formed by the fructose breakdown in the presence of acid.

2.2.7 Enzymes

Additionally, some enzymes found in honey, such as glucose oxidase, invertase, and amylase, seem to have come from honeybees (Molan, 1992). The production of gluconic acid and the antibacterial properties of honeys both depend on glucose oxidase. The invertase enzyme catalyzes the transformation of sucrose, which is acquired from nectar, into the monosaccharide’s fructose and glucose in a ratio of 1:2:1 (Anklam, 1998). Some other enzymes, such acid phosphatase and catalase, are also possesed in some honeys but cancome from plant pollens and nectar.

2.2.8 Phenolic compounds

Quercetin, chrysin, pinocembrin,pinobanksin, kaempferol, galangin, and luteolin are the main flavonoids contained in honey (Kaskonien and Venskutonis, 2010; Dong et al., 2013). Due to an organic carboxylic acid function and the presence of a phenolic ring, the phenolic substances are classified as aromatic acids.

Numerous plant species have phenolic acids in them (Cai et al., 2004). The bioactivity of honey is influenced by the phytochemicals' makeup (Kaskonien and Venskutonis, 2010). Phenolic substances contribute to the honey's antibacterial, anti-inflammatory, and antioxidant properties.

2.2.9 Pigments

Pigments are those chemicals which gives the color of honey. The most important pigments are carotenoids, polyphenols, anthocyanins and xanthopylls that can be divided into lipid soluble pigments and water-soluble pigments. Other compounds that also can contribute to honey color are sugars, minerals and amino acids.

2.2.10 Pollen, propolis and royal jelly

Bees gather nectar and pollen from plants and flowers, providing the hive containing protein for food. Honey frequently contains pollen. Additionally, honey frequently contains tree and plant pollen that was contaminated by wind (Bruni et al., 2015). Carbohydrates, proteins, DNA, nucleic acids, amino acids, lipids, minerals, vitamins, flavonoids, and phenolic compounds are pollen's component substances (Morais et al., 2011). Plant exudates are the source of Propolis, which bees use to seal the hive and provide a membrane against intruders.

Resin makes up 50% of propolis, followed by wax (35%), pollen (5%), essential oils (10%), and other organic components (5%). Propolis contains more than 300 different chemicals, including flavonoids, phenolic compounds, terpenes, esters, and anthraquinones (Kalogeropoulos et al., 2009;). A liquid with proteins is royal jelly. It is exclusively produced by glands in the worker bees' hypopharynx and provided to adult queen bees as a critical source of sustenance (Martos et al., 2008). Major royal jelly proteins (MRJPs), which make up more than 50% of the royal jelly, have been studied and analyzed (Won et al., 2009) to be utilized as a dietary supplement to treat various ailments, such as high cholesterol, seasonal allergies, and asthma.

2.2.11 Hydrogen peroxide

In the 1960s, hydrogen peroxide (H_2O_2), a significant antibacterial component in honey, was discovered. During the oxidation of honey's glucose to oxygen, hydrogen peroxide is naturally formed (Brudzynski et al., 2011). The acidity and sterility of honey are also influenced by hydrogen peroxide.

Inhibition of bacterial growth and DNA destruction are the results of oxidative damage caused by hydrogen peroxide and honey phenolics with pro-oxidant activity (Brudzynski et al., 2011, Brudzynski et al., 2012). Additional research revealed that the concentration

of hydroxyl radicals produced by hydrogen peroxide and its direct relationship to hydroxyl radical formation explained the bacteriostatic action (Brudzynski and Lannigan, 2012). According to certain research, the effects of hydrogen peroxide may be affected by other components of honey (Brudzynski et al., 2011).

2.3 Bee derived antimicrobial peptides

The salivary glands and fat body cells create the cysteine-rich cationic peptides known as "bee derived defensins." These peptides play a role in both individual and social immunity (Klaudiny et al., 2005). Royalisin, which comes from defensin and royal jelly, which comes from the haemolymph, are the two distinct defensins that are both encoded by Defensin-1 (Ilyasov et al., 2012). *Staphylococcus aureus, Bacillus subtilis*, and *Paenibacillus larvae* have all been demonstrated to be sensitive to the antibacterial action of defensin-1 (5.5 KDa) (Kwakman et al., 2010; Bucekova et al., 2014).

This is also the agent responsible for American Foulbrood (AFB), a serious bee disease (Katarina et al., 2002). Honey is unregistered as an antibacterial, but it is registered as a stimulant of wound healing, where it is said to promote tissue regeneration and reduce inflammation.10-40 percent (v/v) honey had a bactericidal effect within 24 hours when tested for in vitro bactericidal activity against *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Streptococcus epidermidis,* and *Pseudomonas aeruginosa*. Characterization of the peptide (defensin-1) and additional elements contributing to this bactericidal effect (Kwakman et al., 2010).Additional protein-based antibiotics have previously been reported, but proteins identification was not performed (Mundo et al., 2004).

2.4 Plant derived antimicrobial phytochemicals

Methylglyoxal (MGO) found in Manuka honey is one example of honey whose antibacterial activity is attributed to plant-derived compounds. Plant-derived phytochemicals perform a significant part in the antibacterial activity of honey. Investigations of the bactericidal properties of honey with special emphasis on Manuka honey, have revealed non-peroxide activity.

Different research teams have analyzed and found plant-derived phenolic chemicals that were separated from honey, although it is unclear what role they played in the overall

activity (Loh et al., 2012). According to research (Kwakman et al., 2010), the number of plant-derived components that contribute to honey's antibacterial activity is too little to detect. However, after extraction, flavonoids and phenolics are thought to be a very promising source of natural medical treatments.

Rubus honey was processed using the techniques Solid phase extraction (SPE) and HPLC analysis to remove phenolic chemicals and antibacterial agents. Honey was used to isolate the flavonoids galangin, chrysin, tectochrysin, pinocembrin, and kaempferol as well as the phenolics caffeic, ellagic acids and p-coumaric, *Pseudomonas aeruginosa*, *Proteus mirabilis* and *Salmonella typhimurium* were just a few of the species that the phenolic extracts from the samples demonstrated antibacterial efficacy against. *Proteus mirabilis* and *Bacillus cereus* were the two species that were most vulnerable (Escuredo et al., 2012). Additionally, the phenolics isolated from Rhododendron honeys from Turkey's Black Sea region were studied for their antibacterial and antioxidant properties. High levels of antibacterial activity against *Proteus mirabilis* and *Pseudomonas aeruginosa* were reported in a study. While individual phenolic chemicals rather than combinations may be responsible for honey's effectiveness, more research is necessary to assess these interactions. Due to a combination of these elements, which frequently cooperate, the minor components of honey exhibit high levels of antibacterial activity.

These plant-derived substances have a great deal of potential for use as medicines for improving human health. The flavonoids, phenolics, and organic acids in honey have been found to operate in a variety of activities, including oxygen quenching, hydrogen donation, radical scavenging, and metal ion chelation, which inhibits bacterial growth (Loh et al., 2012). Phenolic compounds' antibacterial properties should not be disregarded, and phytochemicals can affect honey's antimicrobial properties (Molan, 2001). A combination of peroxide and non-peroxide components may also be suppressing bacterial development (Loh et al., 2011).

These phenolic compounds can be extracted using gas chromatography-mass spectrometry (GC-MS) and thin layer chromatography (TLC), which have both been shown to have antibacterial efficacy against *Helicobacter pylori*. The *Helicobacter pylori*, which causes peptic ulcers and persistent active gastritis, is vulnerable to several South African honey

components. The combination or individual actions of volatile chemicals, such as acetic acid, were what caused the activity (Loh et al., 2012; Loh et al., 2013).

2.5 Antimicrobial efficiency of Honey

Honey has been shown to exert bacteriostatic and bactericidal effects on a variety of bacteria, some of which are harmful, but predominantly against Gram positive bacteria. Hydrogen peroxide, an antibacterial agent, is created by the enzyme glucose oxidase in honey and is broken down by the enzyme catalase. Honey has a poor antibacterial peroxide activity if its catalase activity is high. With various non-peroxide antibacterial agents involved, such as acidic, basic, or neutral chemicals, honey can exhibit both peroxide and non-peroxide antibacterial action.

Therefore, many components including aromatic acids and compounds with various chemical properties, as well as the honey's botanical origin, contribute to its antimicrobial effectiveness. The low pH and high sugar content of honey both contribute to its antibacterial properties. Numerous studies show that bacterial development stops after a specific amount of honey action. The length of the growth inhibitory period increases with honey concentration. However, total growth inhibition is crucial for preventing infections. Rubella and the Herpes virus have both been successfully treated with honey (Al-Waili, 2004). Additionally, it acts as a fungicide on many dermatophytes. According to certain studies, honey has a prebiotic effect, which means that when consumed, it encourages the growth of certain beneficial Bifidus and Lactobacillus bacteria in the stomach. It has been demonstrated that honeys from sourwood, alfalfa, sage, and clover in particular contain prebiotic action. Honeydew honey contains oligosaccharides that have prebiotic properties (Bogdanov, 2011).

2.6 Antibacterial activity of honey

The emission of hydrogen peroxide is one of honey's antibacterial qualities, and some honey also contains additional phytochemical antibacterial components. Because honey has a high sugar content and an osmolarity high enough to prevent microbial growth, it also possesses an antibacterial function (Rakhi et al., 2010). While both the antibacterial properties of honey and hydrogen peroxide were eliminated by light, hydrogen peroxide was the cause of the antibacterial activity of honey. Additionally, according to White and

Subers, honey's glucose oxidase, which produces hydrogen peroxidase, may operate as a bacterial inhibitor. It is commonly known that bacteria and honey both generate a catalase enzyme that destroys hydrogen peroxide. If catalase is active at high hydrogen peroxide concentrations, it is inactive at physiological levels. Honey also contained a second group of light-sensitive, heat-stable antibacterial compounds that prevented the growth of *Staphylococcus aureus, Bacillus subtilis, Bacillus alvei, Escherichia coli, Pseudomonas pyocyanes, Bacillus subtilis,* and *Salmonella typhi*.

Cortopassi-Laurino and Gelli compared the physico-chemical characteristics and antibacterial activity of honey manufactured by Africanized honey bees (*Aphis mellifera*) and *Melliponinae* in Brazil (stingless bees). The seven bacterial isolates tested—Bacillus stearothermophilus, Staphylococcus aureus, Klebsiella pneumoniae, and Pseudomonas aeruginosa—exhibited the maximum susceptibility to both types of honey at a concentration of 5–25 percent, whereas Escherichia coli showed the least susceptibility.

The two samples of honey made by the honeybee (Aphis mellifera) were tested for antibacterial activity using the conventional Well diffusion method. Both honey samples were tested against *Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumoniae, Bacillus subtilis,* and *Escherichia coli* at four concentrations (5 percent, 25 percent, 50 percent, and 100 percent w/v).

There are several reports of honey's bactericidal and bacteriostatic action, and honey's antibacterial qualities can be especially helpful against germs that have already evolved resistance to a number of drugs (Osho and Bello, 2010).

2.7 Antifungal activity of honey

Some types of honey exhibit anti-fungal properties. A comparative method was utilized in which honey was put to culture media with and without starch to see whether there was a synergistic effect of starch on the antifungal activity of honey. Five types of honey were tested for their minimum inhibitory concentration (MIC) using Candida albicans. While the control, bluegum, and fynbos honey only partially inhibited the development of *Candida albicans*, three separate samples of South African honey (wasbessie, bluegum, and fynbos) shown antifungal efficacy against *Candida albicans* (Terrab et al., 2004).

2.8 Antiviral activity of honey

Honey has powerful anti-Rubella properties. The antiviral properties of honey may support the continued use of honey in some modern drugs, such as cough syrups, as well as in traditional medicines from various ethnic communities worldwide (Golob et al., 2005).

2.9 Antioxidant activity of Honey

In addition to other elements including organic acids, vitamins, and enzymes, honey contains a number of phytochemicals that can act as dietary antioxidant sources. The flower source and honey variety have a big impact on these anti-oxidants. The antioxidant content of darker honey is often higher than that of lighter honeys. Oxygen Radical Absorbance Capacity, or ORAC, was used by researchers at the University of Illinois Champaign-Urbana to measure the antioxidant content of 14 unifloral honeys in comparison to a sugar counterpart. There was no antioxidant activity in the sugar analog. The aging and disease processes are aided by free radicals and reactive oxygen species (ROS). Humans can defend themselves against these harmful substances by consuming foods high in antioxidants.

Consuming 1.5 g/kg body weight of buckwheat honey or corn syrup has an impact on the plasma's antioxidant and reducing properties in healthy adult humans. A healthy adult's antioxidant defense system may be strengthened by using honey in some foods instead of standard sweeteners given that the average human consumes more than 70 kg of sweets annually (Gheldof and Engeseth, 2002).

2.10 Methods of measurements of antibacterial activity

In spite of a lack of a thorough understanding of the precise processes underlying these effects, the antibacterial properties of honey have been known in practice for more than a century. Van Ketel gave the first justification for honey's antibacterial properties in 1892. The antibacterial component of honey is known as inhibine, and the amount of dilution to which a given variety of honey retains its antibacterial activity is known as the "inhibine number." These words, which Dold and Witzenhausen first used in 1955, call for the creation of a scale from 1 to 5 that corresponds to honey dilutions in increments of 5%, ranging from 25% to 5% (w/v) (Table 1). The principal antibacterial component in honeys, hydrogen peroxide, was found to be the inhibine (White et al.1963)

Table 2.1: Honey inhibine number and its relationship with honey concentration.

Inhibine number	Bacterial growth	Honey concentration	
		(% w/w)	(% v/v)
5	No growth	6.10	5
4	No growth	11.9	10
3	No growth	17.4	15
2	No growth	22.7	20
1	No growth	27.8	25

Other techniques have been employed to evaluate the antibacterial effectiveness of honey. Numerous techniques, including the broth (micro) dilution test, the well/disk diffusion assay, the agar dilution methods, and the time-kill experiment, can be used to quantitatively evaluate the susceptibility of bacteria to honey.

According to CLSI guidelines, these techniques are frequently employed in microbiological laboratories (Clinical & Laboratory Standards Institute). For instance, the agar diffusion assay technique involves applying a little amount of honey or a honey solution to the center of a well (approximately 6 mm in diameter) drilled into a nutrient agar plate that has already been inoculated with a microbial culture. The honey diffuses out into the agar from its application location while the plate is incubating. The zone of inhibition (ZOI), a clear zone surrounding the honey application site, is a gauge of the honey's effectiveness. The effective antibacterial concentration in this assay, however, may be lower than the concentration given to the agar since honey is diluted during diffusion. In other techniques, the nutrient agar or the nutrient broth in which the bacterial culture is cultivated incorporates honey.

A broth micro- or macrodilution assay is the most popular bacterial susceptibility test. The procedure calls for making two-fold dilutions of honey in broth and transferring them to tubes (macrodilution version) or 96-well microtiter plates (microdilution version). The standardized test microorganisms are introduced into each tube or well before being incubated. A spectrophotometric evaluation of the bacterial growth (change in turbidity) is performed. The minimum inhibitory concentration (MIC) for each variety of honey under study can be established by utilizing a series of various concentrations of honey in the broth or agar. The lowest dose of an antibacterial agent that will suppress the apparent

development of germs after an overnight incubation is known as the MIC and is used to assess an antibacterial substance's in vitro activity (Molan, 1992).

Fluorimetry and spectrophotometric methods for measuring absorbance have higher sensitivity, especially when employed with low honey quantities.

The broth microdilution assay, which measures bacterial growth inhibition spectrophotometrically, is the most suitable technique due to its sensitivity. In addition to the traditional plate count, this approach is typically employed to determine the MIC and MBC values. Additional techniques, such as direct microscopic counts or the evaluation of a growth signal (such as a particular metabolite like lactic acid), are also possible. When comparing results from different approaches, it is crucial to understand that the procedure and scientific judgment will have a significant impact on the outcome (Patton et al. 2006).

Chapter 3: Materials and Methods

3.1 Site and period of experiment

The study was conducted in the Poultry Research and Training Centre (PRTC) Laboratories of Chattogram Veterinary and Animal Sciences University. The study was conducted for a period of six month from 1st January, 2019 to 30th June, 2019.

3.2 Collection of Samples

Nine commercially available honeys were collected from a super shop (QPS, GEC circle, Chattogram). One sample was collected from a person, which was said that original natural honey. The samples are described as below:

1. **Forever bee honey:** This is a product of Spain and distributed by the company Forever Living Products.
2. **Organic Aussiebee:** It is an organic black forest honey. This honey is made in Australia and imported and distributed by the company Discovery Products (BD) Ltd.
3. **Apis Himalaya Honey:** This is an Indian product manufactured by the company Apis India Limited.
4. **Shefah: This** is a product of U.A.E.
5. **Ar-Rafi:** This honey is imported from Dubai and produced by the company AR RAFI FOODS L.L.C.
6. **Young's Bee Hives:** This honey is a Pakistani product, produced by the company YOUNG'S (PRIVATE) LIMITED.
7. **Aussiebee:** This honey is made in Australia and imported and distributed by the company Discovery Products (BD) Ltd.
8. **AL SHAFI:** This honey is produced in Dubai, exported by the company Apis Pure Foodstuff Trading LLC.
9. **7Bahar:** This is a honey with comb. The honey is imported from Turkey by Orbit Trading International. The producer of this honey is Manaviar Gida San.ve Tlc. Ltd.
10. **Raw Natural honey:** This honey is collected from a village in Satkania Upozilla in Chattogram.

3.3 Preparation of samples

Commercially available nine honey samples and a natural honey sample were included in the study. All the commercial honey samples were used in their raw form as found in jar which are commercially processed by the companies. All the samples were first taken in sterilized small plastic bottle in a little amount for easy handling. The 100% concentrations of all the samples were used for the research purpose.

3.4 Test microorganisms

Pure isolated cultures of *Escherichia coli* and *Staphylococcus aureus* were collected from PRTC (Poultry Research and Training Centre), Chattogram Veterinary and Animal Sciences University, Chattogram by using nutrient agar aseptically. Then collected isolates were sub-cultured with selective agar media to obtain more pure isolates.

3.5 Reagents and apparatus

3.5.1 Reagents:

1. 1% Barium Chloride solution
2. 1% Sulfuric acid
3. Normal saline
4. Distilled water

3.5.2 Media:

1. Mueller Hinton agar
2. Nutrient agar
3. Selective agar

3.5.3 Apparatus:

1. Petri dishes
2. Inoculating loop
3. Screw capped test tubes
4. What man no 1 filter paper
5. Volumetric flasks
6. Pipette
7. Beaker
8. Spirit lamp

9. Tripod stands
10. Electric weight machine
11. Foil paper
12. Spoon
13. Marker pen
14. Autoclave
15. Incubator

3.6 McFarland standard preparation

1% Barium Chloride solution and 1% Sulfuric acid were prepared. For 0.5 McFarland standard 9.95ml sulfuric acid and 0.05ml barium chloride solution were mixed in a screw capped sterile test tube.

3.7 Culture suspension preparation

An inoculum of each isolate was prepared from subculture. 4-5 colonies of each isolates were taken in sterilized screw cap tube containing 2ml of sterilized saline water. The bacterial culture was then emulsified in sterile normal saline and the turbidity adjusted to 1.5*10^8 (CFU/ml corresponding to 0.5 McFarland standard).

3.8 Media preparation

Mueller Hinton agar powder 38gm was weighed and mixed with 1L distilled water as described in the label. The media was then boiled to melt and mix properly. After mixing, the media was sterilized in autoclave and kept in water bath for cooling. After cooling, the media poured on the petri dishes aseptically and allowed to consolidate. The dishes were incubated at 37 °C for 24 hours to check if any contamination occur.

3.9 Antimicrobial effect of samples against *Escherichia coli* and *Staphylococcus aureus*

To test the efficacies of the extracts, disc diffusion method was used and its effect was assessed by measuring the zone of inhibition around the disc. Discs of 6 mm diameter were prepared from Whatman No.1 Filter paper. The discs were impregnated with .5ml of each sample. A sterile cotton swab was dipped into the standardized bacterial suspension and used to evenly inoculate the Mueller Hinton agar plates. They were allowed to dry for 3 to 5 minutes. Thereafter, all discs were placed on the plates and µ pressed gently to ensure complete contact with agar. A distance of at least 15mm was maintained from the edges of the plates to present overlapping of inhibition zones. Five samples were placed in a petri dish. Fifteen minutes after the placement of discs, the plates were incubated for 24 hour at 37 ℃. After incubation the plates were examined and diameter of the inhibition zone was measured for each isolate.

Chapter 4: Results

4.1 Antimicrobial activity against *Escherichia coli*

The effect of all honey samples was evaluated individually against *Escherichia coli* using the disc diffusion method. All the samples showed no zone inhibition against *Escherichia coli* (Figure 4.1 and 4.2).

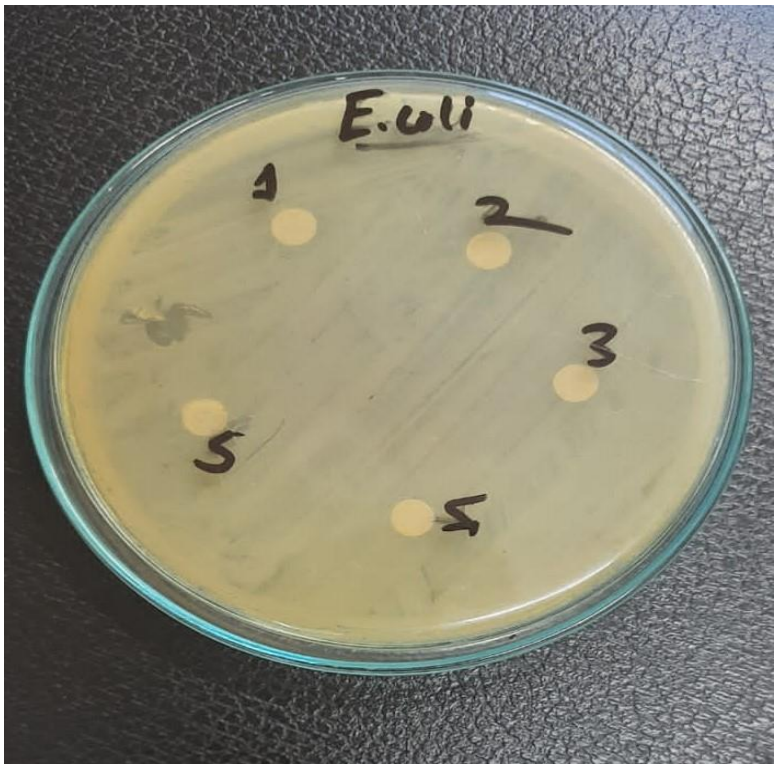

Figure 4. 1: Petri dish with samples 1, 2, 3, 4, 5 with no inhibition zones

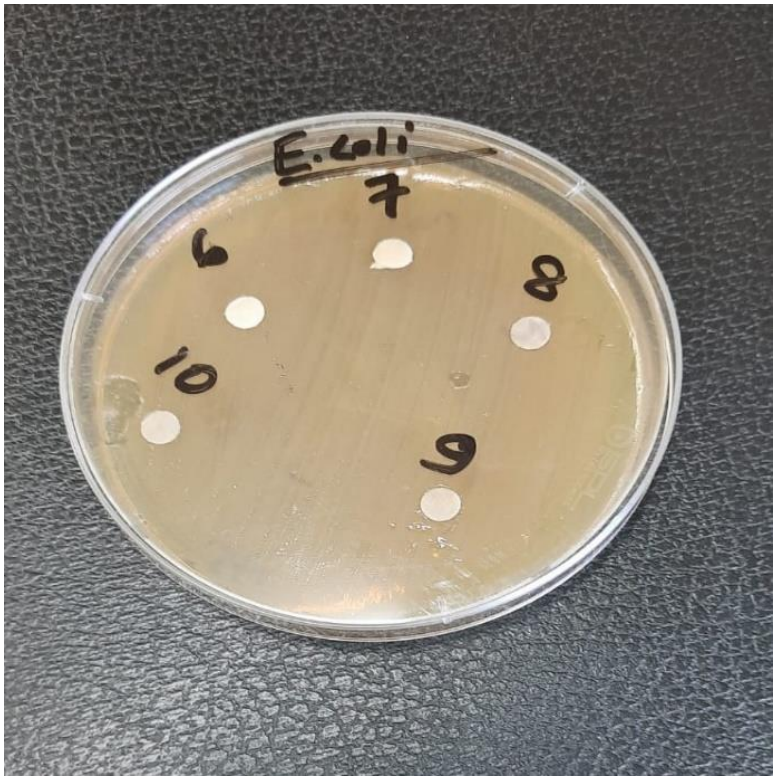

Figure 4. 2: Petri dish with samples 6, 7, 8, 9, 10 with no inhibition zone

4.2. Antimicrobial activity against *Staphylococcus aureus*

The effect of all honey samples was evaluated individually against *Staphylococcus aureus* using the disc diffusion method. All the samples showed no zone inhibition against *Staphylococcus aureus* (Figure 4.3 and 4.4).

Figure 4. 4: Petri dish with samples 1, 2, 3, 4, 5 with no inhibition zone

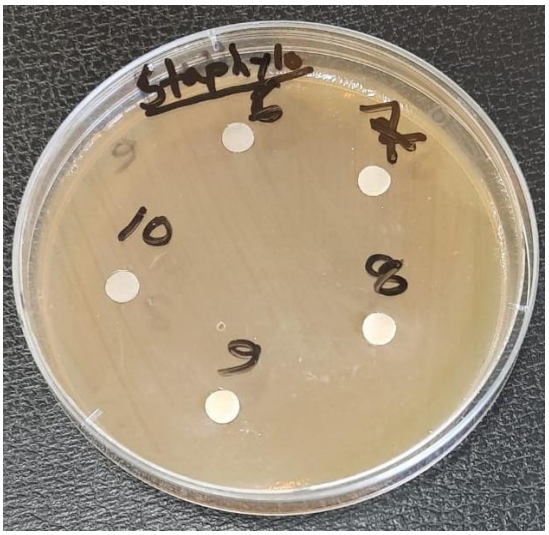

Figure 4. 3: Petri dish with samples 6, 7, 8, 9, 10 with no inhibition zone

Chapter 5: Discussion

In this study, no honey sample exhibited a zone of inhibition against *S. aureus* and *E. coli*. Because of differences in pH, active ingredient content (hydrogen peroxide, antioxidants, phenols, methyl glyoxal, defensin-1, etc.), storage circumstances, and bacterial strain susceptibilities, not all samples have the same antimicrobial effectiveness. Variations in quality and potency, which make it impossible to determine the dosage and formulation, are among the difficult issues with using drugs for medicinal purposes.

There are no rules governing the standardization due to the wide variations in composition, processing methods (such as extraction, filtration, boiling, etc.), and storage conditions, there are currently no guidelines for quantitative assessment of honey's antimicrobial activity.The topic of whether these samples may be used systemically as an alternative to traditional antibiotics has not yet been addressed by controlled, randomized clinical trials of these for usage in a therapeutic environment (Cooper and Jenkins, 2012).

The value of medicinal honey is becoming more widely acknowledged as a high-value product that can be produced commercially in many locations around the world, including in rural and resource-limited settings, as well as a potently active medication that is effective against pathogens that are resistant to antibiotics. However, there is still a lack of knowledge regarding the factors that can ensure the production of honey with therapeutic benefits. Antimicrobial assays are typically conducted on raw, unprocessed honey that has been diluted and filtered to remove microorganisms before testing but has not been heated. However, if the honey needs to be heated afterward to filter out particulate debris, the results may not be accurate.

It was assumed that the generation of H_2O_2 was responsible for the majority or all of the observed activity because the antibacterial activity was reduced to negligible levels.Glucose oxidase, a bee enzyme released by the hypopharyngeal glands, breaks down glucose in honey to create gluconic acid and H_2O_2.A lack of free water and an acidic pH render glucose oxidase inactive, but when the honey is diluted with water, activity is restored. This results in a slow, persistent release of H_2O_2, at levels that are high enough to have an antibacterial impact but low enough to not harm mammalian tissues. Since honey

was not diluted in the current investigation, there was no antibacterial action to speak of. The antimicrobial activity of processed honey samples was, on average, lower.

Enzymes are often adversely affected by heating above physiological temperatures, and a prior investigation on glucose oxidase in honey indicated that heating at 50°C for 20 min drastically decreased enzyme activity (Schepartz and Subers, 1964). Even though H_2O_2 levels were high prior to processing, it's possible that the stability of H_2O_2 production plays a significant role in determining the activity of a honey sample. A honey that loses its capacity to produce H_2O_2 after undergoing standard heat processing may lose useful therapeutic activity. The honey industry would benefit greatly from a test to predict antibacterial activity based on H_2O_2 stability, however H_2O_2 levels alone seem to be a poor predictor of ultimate activity levels, therefore more research is necessary.

The condition of the bees and the caliber of their diet can affect the amount of glucose oxidase in honey. However, honey can also contain catalase, peroxidases, and antioxidants like gallic acid and caffeic acid that can breakdown or H_2O_2 prevent it from harming microbial organisms, so glucose oxidase alone cannot tell how much H_2O_2 is created in a specific honey sample

Additionally, it was just revealed that MGO directly alters several proteinacious chemicals in honey, and if this occurs, it may also have an impact on the activity of glucose oxidase. As a result, the final concentration of H_2O_2 in a given honey sample depends on a number of factors that may be present and active to variable degrees. It is not surprising that the various honey samples behaved to heat treatment fairly differently given that any of them could be impacted by honey processing. It is common practice to heat commercial table honey to a temperature of 45 ℃ in order to speed up the filtration process and remove particle material. However, it is crucial to understand that even very low heat processing might diminish antibacterial action.

Honey's viscosity does not vary significantly above 30°C, so processing honey at lower temperatures should be feasible without causing an appreciable increase in difficulty. Other research has reported a decrease in enzymes, antioxidants, and other phytonutrients after processing, albeit this can again vary greatly between samples.

Furthermore, there is huge practice of adulteration in Bangladesh. The tested samples that are claimed as original products may be artificial honey. These samples should be tested before antimicrobial test whether they are authentic or duplicate adulterated.

Therefore, for honey produced for medical purposes, minimal processing is advised, and samples should be evaluated after processing to verify antibacterial activity is not dramatically diminished.

Chapter 6: Conclusions

We conclude from this study that the examined honeys from renowned company available in Chattogram city possess no antimicrobial activity. In general, processing with heat and filtration reduces H_2O_2-based activity which is correlated with antimicrobial activity of honey but this varies in different honey samples. H_2O_2 stability could be a useful indicator of antimicrobial activity, but further research with a greater number of samples is required to determine the accurate causes of reducing the antimicrobial activity.

When processing and testing honey intended for therapeutic use, one should be aware of the possible harmful consequences of even minimal heating. It is also important to establish standards for using honey in medical purpose.

Before using honey as medicine, one should make sure if the honey is authentic or duplicate.

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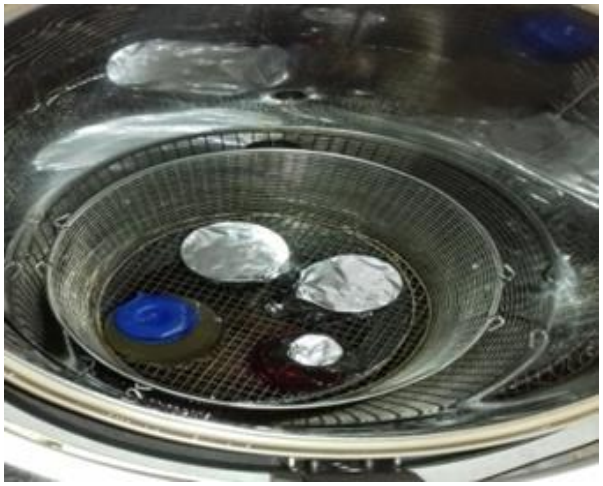
Appendix

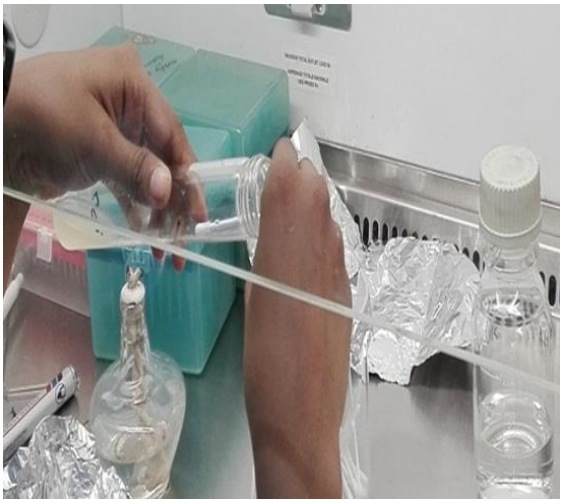
Photo gallery

Commercially available honey samples

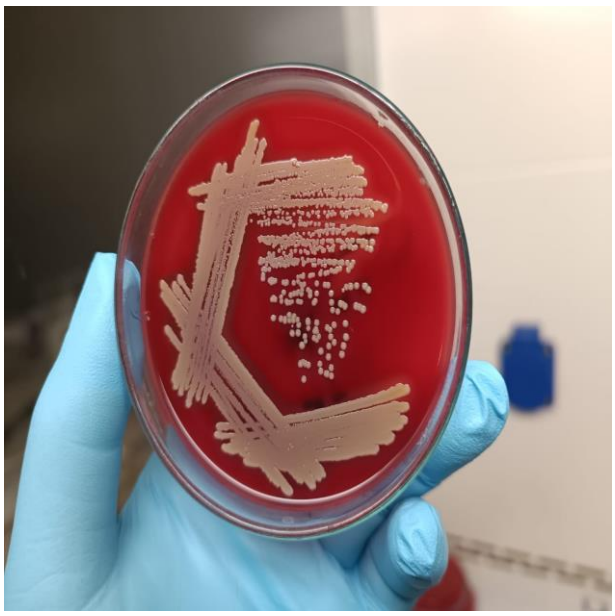
Honey samples

Agar preparation





Sample streaking on agar



Culture of *Staphylococcus aureus*

Culture of *E. coli*

Brief Biography

Fahmida Sultana has successfully finished the B.Sc. in Food Science and Technology program at Chattogram Veterinary and Animal Sciences University's Faculty of Food Science and Technology. She passed the Higher Secondary Certificate Exam in 2012 after passing the Secondary School Certificate Exam in 2010. She is currently a candidate for the Master of Food Chemistry and Quality Assurance degree through the Faculty of Food Science and Technology at Chattogram Veterinary and Animal Sciences University's Department of Applied Chemistry and Chemical Technology. Her research interests include the microbiological safety of food, food that’s used as medicine, creation of new products, food processing, preservation, toxicology, management of food safety and safety, public health, and innovation in the food industry